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Association between Vitamin D Level, Vitamin D Receptor Gene Polymorphisms, and Cathelicidin Level to Acute Lower Respiratory Infections, and the Picture of Exon 2-Vitamin D Receptor Gene Polymorphisms in Children under 5 years old

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Abstract

BACKGROUND: Acute lower respiratory infections (ALRIs) are infectious diseases with high morbidity and mortality in children under five. There are several factors associated with ALRIs (bronchiolitis or pneumonia) that have been established. In recent years, Vitamin D level, Vitamin D receptor (VDR) gene polymorphism, and cathelicidin level are also associated with ALRIs. Until now, there was no VDR gene other than *Fok1* identified at the exon 2-VDR gene.

OBJECTIVE: The objective of this study was to establish whether Vitamin D deficiency, *ff* genotype-*Fok1* VDR gene polymorphism, and low levels of cathelicidin are risk factors of ALRIs and to determine the pictures of exon 2-VDR genes polymorphisms in children under five.

METHODS: A matched case–control study was conducted in children under the age of five. There were 35 subjects who suffered from bronchiolitis or pneumonia and 35 healthy subjects as a control group. These groups were matched based on age and gender, and the children originated from the same neighborhood. Level of 25(OH) D, exon 2-VDR genes sequencing, and level of cathelicidin were investigated. Data were analyzed by the Chi-square test or Fisher exact test and logistic regression with a significant level of $p < 0.05$.

RESULTS: This study found that Vitamin D deficiency and low levels of cathelicidin were risk factors of ALRIs (odds ratio [OR] = 5.82 [95% confidence interval [CI] = 1.71–19.89], p = 0.005 and OR = 4.07 [95% CI = 1.10–15.12], p = 0.036, respectively), while *ff* genotype-*Fok1* VDR gene polymorphism was not (OR = 1.12 [95% CI = 0.26–4.86], p = 1.000). *Fok1* VDR gene polymorphism was the picture of exon 2-VDR gene polymorphisms.

CONCLUSION: It is concluded that Vitamin D deficiency and low levels of cathelicidin are risk factors, but *ff* genotype-*Fok1* VDR gene polymorphism is not a risk factor of ALRIs. *Fok1* VDR gene polymorphism is the picture of exon 2-VDR genes polymorphisms.

Introduction

Bronchiolitis and pneumonia are two of the most common acute lower respiratory infections (ALRIs) in children under the age of five. The World Health Organization estimates about 150 million new cases of bronchiolitis every year, 95% of which occur in developing countries, and approximately 11–20 million (7–13%) require hospitalization [1]. Pneumonia is a high morbidity illness. The worldwide incidence of pneumonia in children under five in 2010 was 120 million, and 14 million of which progressed to severe pneumonia [2]. The mortality rate of bronchiolitis ranged from 0.2% to 7% [1], while the mortality rate of pneumonia is much higher. In 2011, the mortality rate of pneumonia was 1.3 million [3] and 1.1 million in 2013 [4]. Ninety-nine percent of children under five who died due to pneumonia occur in developing countries [5]. In Indonesia, the mortality rate of pneumonia during 2012 was 13.2% [6].

There are several factors associated with ALRIs (bronchiolitis or pneumonia) [7], [8], [9], [10], [11], [12]. Some micronutrients, including Vitamin D, are also associated with ALRIs. Three studies report that Vitamin D is a protective factor of ALRIs [13], [14], [15], while other studies show no association between low Vitamin D intake and bronchiolitis [16], and no association between Vitamin D levels and ALRIs [17], [18].

In addition to low Vitamin D level [13], [14], [15], [19], other factors associated with ALRIs are Vitamin D receptor (VDR) gene polymorphisms [20], [21], [22], and cathelicidin (formed through the role of Vitamin D) level [23], [24], [25]. Different results are shown for VDR gene polymorphisms [26] and cathelicidin level [27].

In addition to inconsistent results, all previous studies were conducted in subtropical countries on white or black ethnic subjects. We know that there is a difference in the time of sunlight exposure between the subtropical and tropical countries [28], and there is a difference in the skin's ability to synthesize Vitamin D due to skin color [29], and there are ethnic variations on VDR gene polymorphisms [30]. Until recently, there has been no study in children simultaneously investigates the association between Vitamin D level, VDR gene polymorphism, and cathelicidin level with ALRIs, and there are no VDR genes, other than *Fok1* that has been identified at exon 2-VDR gene. Therefore, this study was aimed to evaluate, whether Vitamin D deficiency, *ff* genotype-*Fok1* VDR gene polymorphism, and low levels of cathelicidin were risk factors of ALRIs, and to find out the presence of other gene polymorphisms in addition to Fok1 at exon 2-VDR gene.

Methods

This was a case–control study, performed at the Department of Child Health, Udayana University Medical School/Sanglah Hospital Denpasar. This study was conducted from April to October 2015 after approval from the Research Ethics Committee Udayana University Medical School/Sanglah Hospital Denpasar.

Samples for case and control groups were selected by consecutive sampling. We calculated sample size based on α = 0.05 and β = 10% (power 90%) and found that the largest sample for three variables (Vitamin D deficiency, *ff* genotype-*Fok1* VDR gene polymorphism, and low levels of cathelicidin) was 35 for each group. The parent or guardian of each subject gave informed consent. The inclusion criteria for the case group were children under five who suffered from hospitalized bronchiolitis or community-acquired pneumonia, while the inclusion criteria for the control group were healthy children under five who matched the case group in terms of age and sex and came from the same neighborhood. The exclusion criteria for the case group were subjects that suffered from congenital heart disease (CHD), immune deficiency, malnutrition, obesity, cerebral palsy, Vitamin A deficiency, and history of taking medications that contain Vitamin D since the acute respiratory infection (ARI) symptoms begin, or the parent refused to participate, while the exclusion criteria for the control group were the parent refused to participate.

Vitamin D and cathelicidin levels were investigated by enzyme-linked immunosorbent assay methods at Prodia Laboratory. The Vitamin D level was defined as deficient if the calcidiol level ≤20 ng/ml [31], [32], while the cathelicidin level was defined as low if the level of cathelicidin <50 ng/mL [33]. VDR gene polymorphisms were investigated by Sanger's methods at Biochemistry and Genetic Science Laboratories, and the genotypes (*ff, Ff,* and *FF*) were investigated at *Fok1* single nucleotide polymorphisms subject [21]. Bronchiolitis referred to the first episode of acute wheezing in children <2 years of age, starting as a viral upper respiratory infection (rhinorrhea, cough, or low-grade fever) [9], while pneumonia was established based on the presence of fever, cough, tachypnea, respiratory distress, crackles on chest auscultation, and infiltrates or consolidation on a chest radiograph [10], [12]. Subject characteristics and outcomes were initially analyzed by the Chi-square test or Fisher exact test and then continued by multivariable logistic regression analysis for variables that statistically significant at bivariate analysis. We also performed a subgroup analysis for the variable that statistically significant when be tested by bivariate analysis. $p < 0.05$ was considered statistically significant. All data were processed using computer software SPSS 23.0.

Results

During the study period, there were 63 subjects that met the inclusion criteria for the case group and 44 subjects for the control group. For the case group, 28 subjects were excluded due to CHD (three subjects), obese (one subject), parents refused to participate (eight subjects), cerebral palsy (three subjects), immune deficiency (five subjects), malnutrition (six subjects), and two subjects had a history of taking vitamins since the ARI symptoms began. In the control group, nine subjects were excluded due to parents refused to participate in this study. Of the 35 case subjects, we obtained, 22 subjects suffered from bronchiolitis, and 13 subjects suffered from pneumonia.

Overall, we found that 80% of subjects were 2–11 months of age and 63% were male. There were no differences in characteristics between case and control groups except crowded residential environments (Table 1).

Table 1: Characteristics of the study subjects

Variables	Case (n=35)	Control (n=35)	p
1. Age (2–11 months), n (%)	28 (80)	28 (80)	1.000[a]
2. Sex (male), n (%)	22 (63)	22 (63)	1.000[a]
3. Nutritional status, n (%): Underweight+overweight	10 (29)	8 (23)	0.584[a]
4. LBW history, n (%)	6 (17)	4 (11)	0.495[a]
5. Preterm delivery, n (%)	6 (17)	4 (11)	0.495[a]
6. Non-exclusive breastfeeding, n (%)	23 (66)	22 (63)	0.803[a]
7. Non-complete immunization status, n (%)	22 (63)	19 (54)	0.467[a]
8. Indoor air pollution, n (%)	9 (26)	6 (17)	0.382[a]
9. Crowded residential environment, n (%)	14 (40)	3 (9)	0.002[a]
10. Cigarette smoke exposure, n (%)	17 (49)	13 (37)	0.334[a]
11. Asthma, n (%)	1 (3)	-	1.000[b]
12. Day care attendance, n (%)	1 (3)	-	1.000[b]

The only significant p value in this characteristic is the crowded residential environment with p=0.002.
[a]Chi-square test, [b]Fisher exact test.

We found that 27 subjects in the case and eight subjects in control groups were Vitamin D deficiency. Bivariate analysis showed that Vitamin D deficiency was a risk factor of ALRIs. Subgroup analysis found that Vitamin D deficiency was a risk factor for bronchiolitis but questionable for pneumonia (Table 2).

Table 2: Risk of ALRIs, bronchiolitis, and pneumonia in Vitamin D deficiency subject

Case	Control	OR (95% CI)	p
27	8	11.39 (3.73–34.76)	<0.0001[a]
19	5	21.59 (4.46–103.90)	<0.0001[b]
8	3	5.33 (0.97–29.39)	0.047[c]

The analysis showed that Vitamin D deficiency significantly was a risk factor for bronchiolitis but questionable for pneumonia. [a]ALRIs, [b]Bronchiolitis, [c]Pneumonia. ALRIs: Acute lower respiratory infections, OR: Odds ratio, CI: Confidence interval.

After sequencing the PCR of the exon 2-VDR gene using primer reverse, no other genes obtained in addition to the *Fok1* VDR gene. Nine (12.9%) subjects showed *ff* genotype, 25 (35.7%) subjects showed *Ff* genotype, and 36 (51.4%) subjects showed *FF* genotype. We found that both *ff* and *Ff* genotypes were not risk factors of ALRIs compared to the *FF* genotype (Table 3).

Table 3: Risk of ALRIs based on the genotype of *Fok1* VDR gene polymorphisms

Fok1 VDR gene polymorphisms	Case (n=35)	Control (n=35)	OR (95% CI)	p
ff genotype	5	4	1.12 (0.26–4.86)	1.000[b]
Ff genotype	11	14	0.70 (0.25–1.96)	0.500[a]
FF genotype	19	17	1.00 (reference)	-

ff and *Ff* genotypes were not risk factors of ALRIs compared to the *FF* genotype (p>0.05). [a]Chi-square test, [b]Fisher exact test. ALRIs: Acute lower respiratory infections, VDR: Vitamin D receptor.

We found that 25 subjects in the case and seven subjects in control groups were low levels of cathelicidin. Bivariate analysis showed that low levels of cathelicidin were a risk factor of ALRIs. Subgroup analysis found that low levels of cathelicidin were a risk factor for bronchiolitis and pneumonia (Table 4).

Table 4: Risk of ALRIs, bronchiolitis, and pneumonia in low-level cathelicidin subjects

Case	Control	OR (95% CI)	p
25	7	10.00 (3.31–30.23)	<0.0001[a]
16	4	12.00 (2.86–50.31)	<0.0001[b]
9	3	7.50 (1.31–43.03)	0.018[c]

Low levels of cathelicidin were a risk factor for bronchiolitis and pneumonia (p<0.0001). [a]ALRIs, [b]Bronchiolitis, [c]Pneumonia. ALRIs: Acute lower respiratory infections, OR: Odds ratio, CI: Confidence interval.

Logistic regression analysis found that both Vitamin D deficiency and low levels of cathelicidin were risk factors of ALRIs (Table 5).

Table 5: Logistic regression multivariable analysis of ALRIs risk factors

Variables	OR	95% CI	p
Vitamin D deficiency	5.82	1.71–19.89	0.005
Low level of cathelicidin	4.07	1.10–15.12	0.036
Crowded residential environment	2.13	0.41–11.06	0.368

Both Vitamin D deficiency and low levels of cathelicidin were risk factors of ALRIs (p<0.05). ALRIs: Acute lower respiratory infections, OR: Odds ratio, CI: Confidence interval.

Discussion

In children under five, ALRIs are ARI with a fairly high incidence and high mortality rate (especially pneumonia with a mortality rate of 1.1 million in 2013) [4]. There are several factors associated with ALRIs [7], [8], [9], [10], [11], [12]. We found that all of them were comparable in case and control groups except the crowded residential environment (Table 1). The crowded residential environment will facilitate germ transmission from one subject to another subject.

In addition to the role of calcium and bone metabolisms [34], in recent years, Vitamin D is also considered to play an important role in several diseases, including ALRIs. Calcidiol is the best indicator to determine total Vitamin D status in the body, although the biologically active form of Vitamin D is calcitriol [35], [36]. This study found that Vitamin D deficiency was a risk factor of ALRIs. Similar results are shown in other studies [13], [14], [15], [25], [37].

A different result was shown by Roth *et al.* [17] who found that the mean calcidiol levels were similar between case groups (ALRIs patients mainly bronchiolitis) and control (healthy children) groups (77 nmol/L vs. 77.2 nmol/L, p = 0.960). They did not analyze the role of the 1-OHase enzymes, VDR gene polymorphisms, and cathelicidin levels. Another study by Leis *et al.* [16], in a *post hoc* analysis, found that Vitamin D intake <80 IU/kg/day was not a risk factor for bronchiolitis (odds ratio [OR] = 1.7 (95% confidence interval [CI] 0.7–4.0]), but a risk factor for pneumonia (OR = 7.9 [95% CI 1.8–35.5]). The difference in results may be due to the difference in methods. Leis *et al.* [16] only measured Vitamin D intake, they did not measure calcidiol levels. The disruption of enzymes involved in the metabolism of Vitamin D, such as 25-OHase or 1-OHase enzymes, will influence calcidiol or calcitriol production that will also lead to cathelicidin production during adequate Vitamin D intake. In addition, Leis *et al.* [16] did not investigate the role of VDR gene polymorphisms. The difference in the genotype of the *Fok1* VDR gene also influences the incidence of ALRIs [22].

Subgroup analysis of our study found that Vitamin D deficiency has a greater effect size for bronchiolitis than pneumonia. This result may be due to the cause of bronchiolitis, generally virus, especially respiratory syncytial virus (RSV), while pneumonia can be caused by a bacteria or virus. Litonjua [38] stated that Vitamin D reduces the inflammation that occurs after viral (RSV) infection.

VDR gene polymorphisms are also associated with ALRIs. VDR gene instructs the body to make a protein called VDR that will enable the body to respond to Vitamin D [39]. In some ethnicities, VDR is highly polymorphic. There are several polymorphisms that have been reported for the VDR gene; one of them is *Fok1* that is located at exon 2 [40]. Polymorphisms in *Fok1* occur due to changes in the thymine/cytosine (T/C) sequence [41] with basic alteration on codon ATG to ACG at the first potential start site [42]. Based on gene sequencing at exons 2, we found only *Fok1* VDR gene polymorphisms.

The *Fok1* VDR gene consists of three genotypes, that is, *ff, Ff,* and *FF*. The *ff* genotype is less active in transcription the cathelicidin gene and will lead to low cathelicidin levels, and therefore a higher risk to suffer from ALRIs [22]. The short 424 amino acid (aa) VDR protein variant (the "C" allele or "*F*" allele) has greater activity (1.7 times) than long 427 a variant (the "T" allele or "*f*" allele) in its transactivation capacity as a transcription factor [41]. This study found that the *ff* genotype-*Fok1* VDR gene was not a risk factor of ALRIs compared to the *FF* genotype. These results support a previous study that found no difference between VDR gene polymorphisms in children with recurrent tonsillopharyngitis compared to healthy children [26]. Different results were obtained by Roth *et al.* [21] who conducted a case–control study in children aged 1–24 months. They observed that the *ff* genotype-*Fok1* VDR gene was a risk factor of ALRIs (predominant bronchiolitis) compared to the *FF* genotype (OR = 7.38 [95% CI = 1.17–46.55], p = 0.033). The difference in results occurs due to the control subjects. The control subject of our study was healthy subjects during the study period (that perhaps previously had ALRIs), while Roth *et al.* [21] used 1–24 months age subjects that were hospitalized for surgical reasons, without a history of hospitalization due to ALRIs. Another possible cause of the different results is the differences in ethnicity.

Our study also found that low levels of cathelicidin were 4 times more likely to cause ALRIs. Similar results are shown in other studies. Albanna *et al.* [25] performed a case–control study in Egyptian children and found that children with cathelicidin levels <20 ng/mL were risk factor for pneumonia compared to children with cathelicidin levels >20 ng/mL (OR = 10.33 [95% CI = 3.31–33.62], p < 0.001). A study on adults reported higher rates of death from infectious disease in subjects who have low levels of circulating cathelicidin (OR = 3.7 [95% CI = 1.2–11.2]) [24].

Cathelicidin has broad-spectrum antimicrobial activity against microorganisms [43], [44]. Transcription of cathelicidin antimicrobial peptide (CAMP) is increased during bacteria, viruses, fungi, or protozoa infections [45]. The antigen of these agents will activate toll-like receptor-2; this receptor then triggers response mediated by Vitamin D in the form of the interaction between calcitriol and VDR. This interaction will activate CAMP genes to express cathelicidin. This evidence makes Vitamin D as an important factor in the regulation of cathelicidin and can explain the antimicrobial role of Vitamin D [33], [46].

In contrast to our study, Leow *et al.* [27] performed a cohort study in adults with pneumonia that was selected by convenient sampling and found that cathelicidin and β-defensin-2 cannot predict mortality. Differences in results may be due to differences in age (children are not representative of adults), outcomes, and sample selection.

On subgroup analysis, we found that low levels of cathelicidin have a greater effect size in for bronchiolitis than pneumonia. This result was consistent with the results of the subgroup analysis for Vitamin D levels. Some studies have shown a positive correlation between the levels of Vitamin D and cathelicidin [25], [45], [47]. Greater effect size in bronchiolitis than pneumonia is possibly due to the proportion of the virus causing bronchiolitis that is greater than the proportion of the virus causing pneumonia. Miller *et al.* [48] reported that the causes of bronchiolitis are RSV (76%), human rhinovirus (18%), influenza virus (10%), human metapneumovirus (3%), coronavirus (2%), and parainfluenza virus (PIV) 1%, while only 30% of pneumonia are caused by viruses [49], of which 20% due to RSV, 3% by the influenza virus, and 7% due to PIV [10]. RNA virus will increase active Vitamin D on epithelial cells of the respiratory system and expression of cathelicidin (this does not happen on bacterial antigen wall component). Vitamin D will upregulate cathelicidin expression in the epithelial cells of the respiratory system and plays an important role in host defense. Vitamin D also activates the secretion of β-defensin-2 from neutrophils. Beta-defensin-2 has activity against RSV, influenza virus, PIV, and adenovirus [50].

As far as we know, this is the first study that simultaneously identifies the association between Vitamin D deficiency, *Fok1* VDR gene polymorphism, and low levels of cathelicidin with ALRIs in children under five. This study also found Vitamin D deficiency and low levels of cathelicidin as a risk factor for ALRIs with a different cut off point to the previous studies and also got a greater effect size for bronchiolitis than pneumonia.

This study had several limitations, such as control patients not being assessed for history of ALRI, infection cause (viral or bacterial) not being investigated, and calcium, parathyroid hormone, and 1-OHase levels not being measured.

Conclusion

Finally, we conclude that Vitamin D deficiency and low levels of cathelicidin are risk factors, but *ff*-genotype-VDR gene polymorphisms are not a risk factor of ALRIs. Subjects with Vitamin D deficiency or low levels of cathelicidin have a greater risk of suffering from bronchiolitis than pneumonia. *Fok1* VDR gene is the only picture of exon 2-VDR gene polymorphisms. To prevent Vitamin D deficiency, we suggest that sun exposure during the daytime is essential (4–10 min for white ethnics, 60–80 min for black ethnics, and among for brown skin) [29], children under five must have a diet that contains 400 IU/day of Vitamin D [31] and occasionally measure Vitamin D levels in children. Further studies are needed to examine calcium,

parathyroid hormone, and 1-OHase enzyme and the control selection using children that have never suffered from ALRI.

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